Transient adenoviral *N*-methylpurine DNA glycosylase overexpression imparts chemotherapeutic sensitivity to human breast cancer cells

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Abstract

In an effort to improve the efficacy of cancer chemotherapy by intervening into the cellular responses to chemotherapeutic change, we have used adenoviral overexpression of *N*-methylpurine DNA glycosylase (MPG or ANPG/AAG) in breast cancer cells to study its ability to imbalance base excision repair (BER) and sensitize cancer cells to alkylating agents. Our results show that MPG-overexpressing cells are significantly more sensitive to the alkylating agents methyl methanesulfonate, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, methylnitrosourea, dimethyl sulfate, and the clinical chemotherapeutic temozolomide. Sensitivity is further increased through coadministration of the BER inhibitor methoxyamine, which covalently binds abasic or apurinic/apyrimidinic (AP) sites and makes them refractory to subsequent repair. Methoxyamine reduction of cell survival is significantly greater in cells overexpressing MPG than in control cells, suggesting a heightened production of AP sites that, if made persistent, results in increased cellular toxicity. We further explored the mechanism of MPG-induced sensitivity and found that sensitivity was associated with a significant increase in the number of AP sites and/or single-strand breaks in overexpressing cells, confirming a MPG-driven accumulation of toxic BER intermediates. These data establish transient MPG overexpression as a potential therapeutic approach for increasing cellular sensitivity to alkylating agent chemotherapy. [Mol Cancer Ther 2004;3(8):955–67]

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Introduction

The majority of chemotherapeutic drugs currently used to treat cancer by inducing changes in the DNA do so by reacting through oxidation or alkylation reactions to cause chemical modifications of the DNA bases (1). One of the major cellular mechanisms that has evolved to deal with these types of adducts is the DNA base excision repair (BER) pathway (2). Because it is responsible for handling lesions that result from a large number of chemotherapeutic drug regimens, BER is an obvious candidate for intervention into the cellular responses to DNA change (3). BER is responsible for the recognition and removal of DNA modifications that result from chemical hydrolysis, oxidation, and alkylation of DNA largely by reactive intracellular metabolites and also by external and environmental agents such as chemotherapeutics. Because of the frequency and spectrum of DNA base damage recognized and repaired, BER can be considered one of the most significant of the DNA repair pathways (4).

In BER, adducted bases are singly removed from the DNA and replaced by normal residues in a multistep process that relies on duplex redundancy as a template. Several discrete enzymes are sequentially involved in repair, beginning with a lesion-specific glycosylase that recognizes and releases the damaged base resulting in a baseless or abasic or apurinic/apyrimidinic (AP) site. Glycosylases catalyze base excision from the DNA by cleaving the glycosyl bond between the damaged base and its corresponding ribose residue, producing an AP site as the first step in repair. Unrepaired AP sites block DNA (5, 6) and RNA polymerases (7), stalling both replication and transcription. Polymerase blocking interruption of the vital cellular process of replication is a potent cause of double-strand breaks and cell death (8). Polymerase arrest can, on occasion, be overcome by lesion bypass and template-independent base insertion, and this can lead to mutagenesis resulting from the incorporation of incorrect bases (9–11).

AP sites, whether they are produced as intermediates in BER pathways or as the result of spontaneous base loss, are processed by the indispensable enzyme activity of APE1, which introduces a strand break into the DNA backbone 5′ to the baseless site (12). The resulting single-strand break represents a further disturbance of the DNA structure that interrupts replication and transcription and, when unrepaired, leads to cell death (13). Adjacent breaks on opposing strands and those present during DNA replication are converted into double-strand breaks (14), which constitute the most significant form of DNA damage, known to strongly promote the signal toward cell death (15). Thus, this system of excision repair involves processing modest structural DNA damage through baseless and

strand-interrupted intermediates to reinstate the correct base structure. These intermediates represent profound alterations of DNA integrity that are cytotoxic when left unrepaired.

In early research aimed at understanding the significance of initiating lesions removed by BER, Kaina et al. stably overexpressed the human enzyme *N*-methylpurine DNA glycosylase (MPG) in Chinese hamster ovary cells (16). This enzyme is a broad substrate recognition glycosylase that excises a wide variety of damaged bases such as N^3-methyladenine, N^7-methylguanine, N^3-methylguanine (17, 18), hypoxanthine (19), 1,N^6-ethenoadenine (20), 3, N^3-ethenoguanine, and 8-oxoguanine (21). Surprisingly, Chinese hamster ovary cells overexpressing 2- to 12-fold increased MPG activity were not resistant to DNA alkylation (16) but paradoxically were more sensitive (22). These cells had increased sister chromatid exchanges, stronger inhibition of DNA replication, higher numbers of DNA breaks, and slightly decreased cell survival (22). These data led to the ''imbalanced repair hypothesis,'' which theorized that MPG overexpression was causing pronounced production of AP sites and/or strand-broken DNA following alkylation (23, 24). Increased MPG is believed to effectively ''outrun'' the downstream rate-limiting step (25) that would appropriately handle toxic BER intermediates and repair the DNA. Rapid high-level production of toxic intermediates leads to inhibition of DNA replication, chromosomal aberrations, and eventual cell death.

We are interested in imbalancing BER to sensitize tumor cells to current alkylating agent chemotherapy. Previously, we have stably overexpressed MPG in breast cancer cells, and this overexpression imparted moderate sensitivity to the alkylating agent methyl methanesulfonate (MMS; ref. 24). Confounding was a dramatic decrease in cell survival in the absence of alkylation. MPG is known to promiscuously remove undamaged normal bases from DNA, and this low level of inappropriate repair has been shown to correlate with enzyme expression level (26). An accruing release of normal bases and/or endogenous alkylated bases over the time of clonal selection is theorized to have caused the decreased cell survival of untreated stable MPG-overexpressing cells (24). This decreased survival of overexpressing clones in the absence of exogenous alkylation raises the concern that some of the effects of MPG overexpression may have been lost or blunted over the course of clonal selection and expansion.

To fully assess the ability of MPG overexpression to sensitize cells to alkylation without the cumulative toxic effects of long-term overexpression and resultant loss of phenotype, we have employed an adenoviral-based transient overexpression system to achieve high-level MPG gene delivery into human breast cancer cells. Our results show that MPG-overexpressing cells are significantly more sensitive to the alkylating agents MMS, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), methylnitrosourea (MNU), dimethyl sulfate (DMS), and the clinical chemotherapeutic temozolomide. Sensitivity is further increased through coadministration of the BER inhibitor methoxyamine, which covalently binds AP sites and makes them refractory to subsequent repair. Methoxyamine reduction of cell survival is significantly greater in cells overexpressing MPG than in control cells, suggesting a heightened production of AP sites that, if made persistent, results in increased cellular toxicity. This increased sensitivity was associated with a significant increase in the number of AP sites and/or single-strand breaks in overexpressing cells, confirming a MPG-driven accumulation of toxic BER intermediates. Baseless sites therefore have profound cellular consequences, and their efficient repair is important to avoid cell death. These data establish transient MPG overexpression as a potential therapeutic approach for increasing cellular sensitivity to alkylating agent chemotherapy.

Experimental Procedures

Cell Lines and Culture Conditions

Human mammary gland adenocarcinoma cell lines MDA-MB-231 were purchased from the American Type Culture Collection (Manassas, VA). Cells were maintained as adherent monolayer cultures in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT) and 1% penicillin/streptomycin (Invitrogen). Cultures were grown at 37°C in a 5% CO_2 water-humidified chamber (Forma Scientific, Marietta, OH) and subcultured prior to confluence. Cells were grown without antibiotic every third or fourth passage to ensure the absence of contamination, and antibiotic was generally excluded from cells during the experiments. Cells were split <25 times before being replaced by fresh frozen stocks.

Vector Construction

Human MPG had been cloned previously from HT-29 cells by reverse-transcription PCR and ligated into the pcDNA3.1 vector (24). To distinguish exogenous transgene overexpression from endogenous MPG gene levels, a hemagglutinin epitope (HA) tag was added to the 5′ terminus using a PCR primer (5′-C**CGAATT**CAT*GTACCCATACCGATGTGCCAGATTACGC*TGTCACCCCCGCTTTGCAG-3′), which contained an *Eco*RI site (bold) and code for the nine–amino acid HA tag (italics). A *Bam*HI-containing(bold) oligonucleotide was used as the 3′ primer: 5′-G**CGGATC**CTCAGGCCTGTGTGTCCTG-3′. This PCR product was cloned into the pCR2.1 vector (TA cloning kit; Invitrogen). The HA tag and entire MPG sequence were confirmed using the DNA sequencing facilities at the Biochemistry Biotechnology Facility at Indiana University School of Medicine (Indianapolis, IN). This HA-MPG and a previously constructed mitochondrial-targeted MPG (24) with the 72-bp targeting sequence from the human manganese superoxide dismutase enzyme (27, 28) were ligated into a modified adenoviral shuttle vector. The pAd5 CMV K-NpA shuttle vector from the University of Iowa Gene Transfer Vector Core Laboratory (Iowa City, IA) was altered to contain an internal ribosomal entry site–driven enhanced green fluorescent protein (IRES2-EGFP; BD Biosciences Clontech, Palo Alto, CA) located between *Bam*HI and *Not*I restriction sites.

Production of Recombinant Adenovirus

The HA-MPG-containing adenoviral shuttle vector along with an empty vector control (pAd5 IRES2-EGFP) were sent to the University of Iowa Gene Transfer Vector Core Laboratory for production and titering of replication-defective recombinant adenovirus (29). Titers ranged from 3×10^{10} to 1×10^{11} infectious units/mL. Resultant adenoviruses (Ad5 IRES2-EGFP and Ad5 HA-MPG) were aliquoted and stored at −80°C until use.

Adenoviral Infection

MDA-MB-231 breast cancer cells growing in 10 cm plates at 60% to 90% confluence were washed with PBS and trypsinized for 10 to 15 minutes at 37°C in a 5% CO_2 water-humidified chamber. Cells were resuspended in a known volume of RPMI supplemented with 10% fetal bovine serum, and viable cells were counted in quadruplicate using a hemocytometer and trypan blue exclusion. Cell suspensions were rocked to prevent settling during counting. Identical cell suspension volumes of >95% trypan blue–excluding cells were pelleted at 5×10^6 to 6×10^6 cells per pellet, and the medium was removed. Cells were resuspended in PBS, and purified adenovirus was added to the suspension at a multiplicity of infection (MOI; ref. 30) of 15 infectious units/cell and a final infection volume of 50 μL. Adenoviral infections were carried out for 3 hours at 37°C in a 5% CO_2 water-humidified chamber and gently agitated every 20 to 30 minutes. Infected cells were resuspended in 10 mL of 10% RPMI and mixed thoroughly before known cell numbers were plated for downstream applications. Infection efficiencies of transduced cells were determined 24 hours after infection by measuring EGFP fluorescence intensities of 10^4 cells using a FACSCalibur fluorescence-activated cell sorter (Becton Dickinson, San Jose, CA) and analyzing results with CellQuest 3.3 software (Becton Dickinson). EGFP expression levels were also visualized by fluorescence microscopy using a TS100F microscope (Nikon, Tokyo, Japan) outfitted with a fluorescence system (Nikon).

Western Blot Analysis

To show adenoviral-mediated overexpression, 1.0×10^6 to 2.0×10^6 infected cells were washed and pelleted at the time of drug treatment and stored at −80°C until needed. Pellets were thawed and resuspended at 1.0×10^7 cells/mL in 1.1× NuPAGE LDS sample buffer (Invitrogen) and boiled for 10 to 15 minutes. Protein concentration was quantified using the detergent-compatible (31) Lowry-based protein assay (Bio-Rad Laboratories, Hercules, CA). One-tenth volume of reducing agent (0.5 mol/L DTT) was added to each sample just prior to loading 20 μg of each into a 10% Bis-Tris NuPAGE gel. Samples were resolved on the gel at 200 V for 35 minutes and transferred to nitrocellulose at 30 V for 1 hour. The membrane was probed with monoclonal MPG antibody (kindly provided by Dr. Rabindra Roy, University of Texas Medical Branch, Galveston, TX) followed by horseradish peroxidase–conjugated rabbit anti-mouse IgG (Chemicon International, Temecula, CA). Antibody binding was detected using chemiluminescence (Roche Diagnostics Corp., Indianapolis, IN), and equal loading was confirmed by probing with pan-actin monoclonal antibody (Neo-Markers, Inc., Fremont, CA).

Immunohistochemistry

Immunohistochemistry was used to visualize HA-MPG overexpression on an individual cell basis within the adherent cellular monolayer and to generally assess subcellular localization. Adenoviral-infected cells were washed with PBS and fixed 24 hours after infection with HistoChoice tissue fixative (Amresco, Solon, OH). Fixed cells were rinsed with TBS and permeablized with ice-cold 0.1% Triton X-100 in TBS. Nonspecific binding was blocked with 10% normal goat serum in TBS for 30 minutes, and cells were incubated with rat anti-HA monoclonal antibody in 10% normal goat serum for 3 hours in a humidified chamber. After incubation with primary antibody, cells were washed with TBS and incubated with biotinylated goat anti-rat IgG in 10% normal goat serum for 1 hour. After rinsing, the slides were incubated for 30 minutes in Vectastain ABC complex (Vector Laboratories, Burlingame, CA) and washed twice before incubating with 3,3′-diaminobenzidine peroxidase substrate (Vector Laboratories) until signal developed.

Oligonucleotide-Based Assay for MPG Glycosylase Activity

To assay HA-MPG-overexpressing cells for MPG glycosylase activity, a previously described fluorometric oligonucleotide-based activity assay was employed (32). Both substrate and product bands were detected and quantified using the FMBioII fluorescence imaging system and software (Hitachi Genetic Systems, South San Francisco, CA).

Drug Treatment

Cells infected with recombinant adenovirus carrying MPG constructs or vector control were treated with DNA-damaging agents 24 hours after the completion of the 3-hour adenoviral exposure, at the time when near-peak transgene levels were expressed. Infected cells were treated with MMS, MNNG, MNU, DMS, methoxyamine (all from Sigma Chemical Co., St. Louis, MO), and temozolomide (a kind gift from Dr. Robert Bishop, Schering-Plough Corp., Kenilworth, NJ). Drug treatment was carried out for 1 hour at 37°C in 5% CO_2. After treatment, drug-containing medium was removed and replaced with fresh growth medium. Methoxyamine experiments included methoxyamine in the medium during and after alkylator treatment. Cells were incubated for another 24 to 48 hours before being analyzed for survival.

Cell Survival/Proliferation Assays

Cell survival of HA-MPG-overexpressing breast cancer cells following DNA-damaging agent exposure was measured 24 and 48 hours after treatment using several different survival assays. Vector-infected cells were used as the control in these experiments, and survival data were normalized to the survival of untreated vector control cells. It should be noted that in all experiments untreated HA-MPG-overexpressing cells had nearly identical survival to untreated vector controls (data not shown).

Three different assays were used for cell viability, survival, and cellular proliferation. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay was used for cell viability. The sulforhodamine B (SRB) assay, which is a commonly used high-throughput assay used by National Cancer Institute, was used for cell survival and growth (33–35), and cellular proliferation was measured using the [^{3}H]thymidine incorporation assay (36).

Assessment of Apoptosis

For analysis of apoptosis, adenoviral-infected cells were treated for 1 hour with alkylating agents as described above. Cell density at the time of treatment was equivalent to that used in the cytotoxicity experiments. Apoptotic cell death was measured by flow cytometry at various times after treatment using phycoerythrin (PE)-conjugated Annexin V and 7-amino-actinomycin D (7-AAD; BD Biosciences PharMingen, San Diego, CA).

Alkaline Comet Assay Analysis

The single-cell gel electrophoresis or comet assay (37, 38) was used to determine the extent of DNA damage in individual adenoviral-infected MDA-MB-231 breast cancer cells treated with MMS. The assay was done according to Trevigen's comet assay protocol (Gaithersburg, MD). Cells that had been infected with IRES2-EGFP or HA-MPG-containing recombinant adenovirus were treated with MMS 24 hours after the completion of infection using identical conditions to those employed in the cytotoxicity experiments. Six hours after MMS exposure, before apoptosis could be detected by Annexin V/7-AAD analysis, cells were washed, trypsinized, and collected for comet analysis.

Comets were silver stained using the Silver Stain Plus protocol (Bio-Rad Laboratories), visualized, and scored from digital photographs (DC120 Zoom Digital Camera, Eastman Kodak, Rochester, NY) of transmission light microscopic images (Nikon Optiphot 2). The distance of DNA migration from each cell was measured from the body of the nuclear core to the trailing edge of the comet using Adobe Photoshop 6.0 (Adobe, San Jose, CA). The comet lengths of 50 individual cells were measured for each treatment group.

Results

Stable overexpression of the BER glycosylase MPG has been shown to sensitize cells to the clastogenic and cytotoxic effects of alkylation (16, 22, 24). This sensitivity is believed to result from unbalanced BER, wherein increased levels of MPG remove alkylated bases from the DNA beyond the capacity of downstream enzymes to complete repair. As a result, unprocessed repair intermediates, such as AP sites and single-strand breaks, lead to double-strand breaks, chromosome aberrations, and cell death.

To transiently overexpress MPG in breast cancer cells, a bicistronic adenoviral vector was constructed to overexpress HA-MPG and an IRES2-EGFP so that infected cells could be distinguished by fluorescence. This allows adenoviral infection efficiencies to be measured and infected cells to be assessed or isolated immediately prior to experimentation, drastically shortening the time for selection of overexpressing cells (39).

Recombinant nonreplicating adenovirus was used to infect MDA-MB-231 breast cancer cells. A MOI (30) or virus-to-cell ratio of 15 was found to infect >75% of the cells within 24 hours of a 3-hour adenoviral exposure as monitored by EGFP fluorescence (flow data not shown). These infection conditions confer high-level MPG gene expression to infected cells that can be detected within 10 hours of infection and approach near-peak levels by 25 hours (Fig. 1A). This level of adenoviral HA-MPG overexpression does not affect cell survival or proliferation when compared with either IRES2-EGFP-infected or uninfected controls by MTS, SRB, and [^{3}H]thymidine assays (data not shown).

To visualize overexpression on an individual cellular basis and to observe subcellular localization of HA-MPG, MDA-MB-231 cells infected with 15 MOI of adenovirus were subjected to immunohistochemistry staining of the HA tag 24 hours after infection. HA immunohistochemistry showed clear HA-MPG overexpression that varied

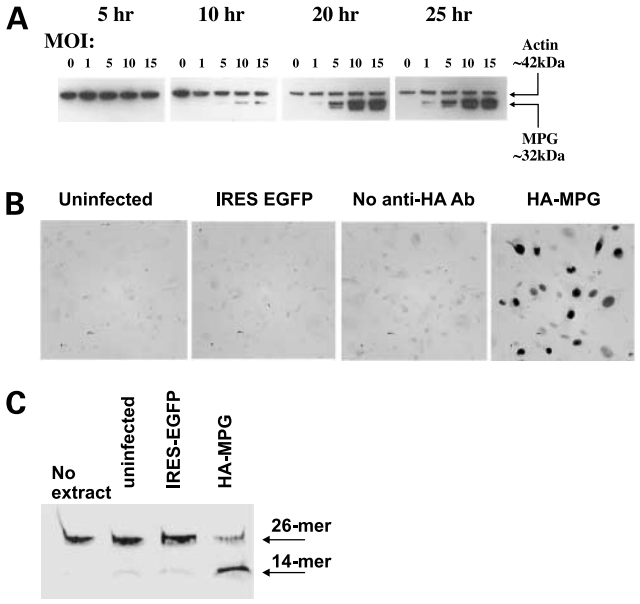

Figure 1. Overexpression of MPG in MDA-MB-231 cells. **A,** anti-MPG Western blot analysis of MDA-MB-231 cells at 5, 10, 20, and 25 hours after infection with 0, 1, 5, 10, and 15 MOI of adenovirus. These infection conditions were used for subsequent overexpression experiments, and similar overexpression profiles were invariably obtained. The *lower band* is a MPG degradation product due to its overexpression in cells. **B,** immunohistochemistry analysis using anti-HA antibody of MDA-MB-231 cells at 24 hours following infection using a 15 MOI for the vector control or HA-MPG adenovirus. Panels include uninfected cells, vector-infected cells, and HA-MPG-infected cells with no primary anti-HA antibody and with anti-HA antibody, respectively. **C,** activity assay demonstrating significantly increased MPG activity in the cell extract of HA-MPG-overexpressing cells. The removal of an εA substrate base in an oligonucleotide duplex followed by alkali cleavage of the labile site converts a 26-nucleotide substrate into a 14-mer product. Bands are detected in a denaturing polyacrylamide gel by virtue of a 5′ fluorescent HEX label.

somewhat within the population of infected cells (Fig. 1B). Significantly, the HA signal was concentrated in the nucleus of infected cells, demonstrating that HA-MPG is not only highly overexpressed but also concentrated correctly in the nucleus of adenovirus-infected cells.

To be sure that overexpressed HA-MPG has enzymatic activity and is therefore capable of imbalancing BER in the presence of alkylation damage, a fluorescent oligonucleotide-based activity assay was used to measure *in vitro* activity of HA-MPG isolated from overexpressing cells (32). MDA-MB-231 cells were isolated 24 hours after a 15 MOI adenoviral infection. Adenovirus-infected cells were assayed for MPG glycosylase activity by incubating extracted protein with a fluorescently labeled oligonucleotide containing a centrally located A, a substrate for MPG glycosylase activity (32). Active MPG is able to remove the A, and alkali generates a shorter-labeled oligonucleotide from the longer starting material. These activity assays showed dramatically increased MPG activity in protein derived from HA-MPG-overexpressing cells compared with uninfected or vector-infected cells, which had very little endogenous MPG activity under these conditions (Fig. 1C). This shows that adenoviral delivery of HA-MPG into MDA-MB-231 cells is an effective means of establishing transient, high-level MPG overexpression that is present in the nucleus and possesses glycosylase activity. This system is capable of achieving high levels of MPG without the discriminatory effects of clonal selection or the mutagenic sequelae of long-term overexpression as has been shown previously (24).

Sensitivity of HA-MPG-Overexpressing Cells to Alkylating Agents

With adenoviral HA-MPG transgene expression as the setting in which imbalanced BER was to be established, we attempted to overload repair by introducing substrate alkylation damage. The model of imbalanced repair relies on heightened MPG activity to remove damaged bases beyond downstream enzyme capacity, but implicit in the model is the corequirement for high levels of alkylated adduct that can be processed by the overexpressed glycosylase but not processed by subsequent enzymes. To deliver increased levels of DNA alkylation damage into MPG-overexpressing cells and imbalance BER, the laboratory alkylating agent MMS was used. MMS is a monofunctional methylating agent that readily reacts with cellular DNA in a second-order S_N2-type reaction. MMS primarily methylates DNA at N^7-guanine and N^3-adenine nucleophilic centers to form 82% N^7-methylguanine, 10.8% N^3-methyladenine, and 0.6% N^3-methylguanine lesions that are all repaired by MPG. MMS also induces a small fraction (0.3%) of the O^6-alkylguanine DNA-alkyltransferase–repaired O^6-methylguanine adduct.

To ensure that cell survival following BER imbalance was accurately assessed, three different assays were used to measure cellular survival following MMS exposure (MTS, SRB, and [^{3}H]thymidine incorporation assays). The MTS assay measures the capacity of viable cells to metabolize MTS. Cell survival measured by the MTS assay includes not only actively proliferating cells but also all metabolically active cells and is based on the assumption that dead cells do not reduce tetrazolium. The SRB assay measures the binding of SRB dye to total protein in cultured cells. SRB binds to basic amino acid residues under mildly acidic conditions so that colorimetric determination of bound dye at 565 nm gives a sensitive determination of total protein mass. The number of total cells remaining following drug treatment can be inferred from this measure of biomass including any residual viable or nonviable cells (33–35). The use of this technique in estimating cell survival relies on the assumption that dead cells are lysed or removed during the procedure, and it may therefore be prone to overestimating the surviving fraction. Because it includes all cells remaining after drug treatment, the SRB assay gives a conservative estimate of cell death resulting from imbalanced excision repair. Finally, the [^{3}H]thymidine incorporation assay measures the insertion of a radiolabel into replicating DNA of proliferating cells. Because DNA replication is required for cell division, [^{3}H]thymidine incorporation gives an indication of cell proliferation after drug treatment (36).

These different assays were used to measure the effect of HA-MPG overexpression on breast cancer cell responses to MMS alkylation. MTS and SRB assays were carried out 24 and 48 hours after MMS exposure, whereas 24-hour long [^{3}H]thymidine incorporation was initiated between 36 and 48 hours after drug treatment. MTS survival assay results of >10 independent experiments showed that HA-MPG-overexpressing cells were significantly more sensitive to the cytotoxic effects of the laboratory alkylating agent MMS (Fig. 2A). This result was verified by both SRB and [^{3}H]thymidine assays (data not shown), which produced nearly identical results. The MPG-induced sensitivity seen in these experiments was much greater than had been shown previously, with stable overexpressing clones treated with lower-dose MMS. Adenoviral-infected HA-MPG-overexpressing cells had a 2.58-fold decreased IC_{50} compared with the 1.53-fold decreased IC_{50} seen previously (24). This experimental system involving high-level transient overexpression and subsequent high-dose MMS treatment represents a significant improvement over previous attempts to imbalance BER.

In addition to demonstrating a significant increase in cell killing by adenoviral MPG-infected cells, we also determined that the level of cell killing is directly proportional to the amount of MPG in the cells (Fig. 2B and C). MTS assays were done as described previously but with varying MOI levels from 1 to 25. As shown in Fig. 2B, as the MOI increases, so does the level of cell killing. The MPG data were compared with vector control infected cells at a MOI of 15 as a control for adenoviral infection effects. Infection levels at MOI 1, 5, 10, 15, and 25 were 24%, 62%, 75%, 84%, and 89% respectively.

Sensitivity of HA-MPG-Overexpressing Cells to Additional Alkylating Agents

In an effort to begin to understand the scope of MPG-induced sensitivity, adenovirus-infected overexpressing

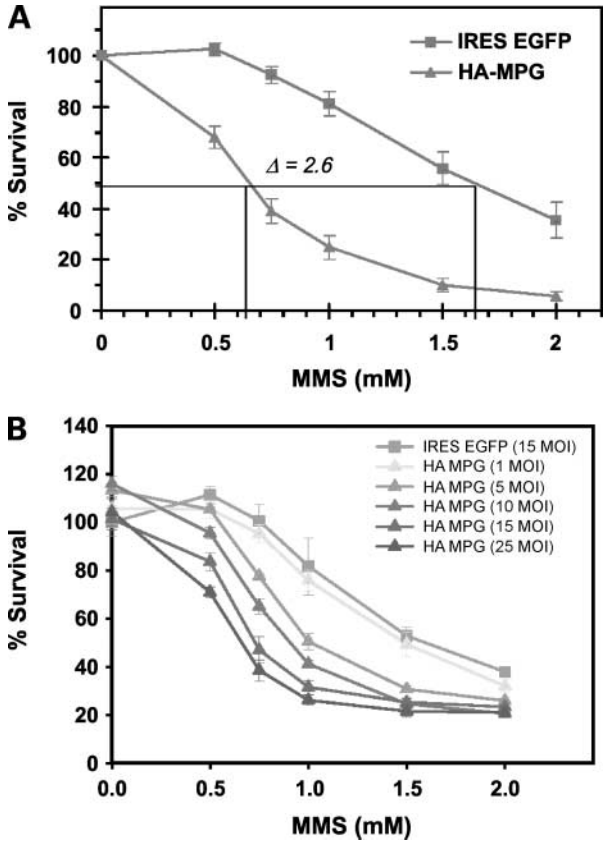

Figure 2. MTS survival assay results of adenovirally infected MDA-MB-231 breast cancer cells treated with MMS. **A,** HA-MPG-overexpressing cells are significantly more sensitive than vector-infected controls (decreased IC_{50} of 2.58-fold) to alkylation by MMS. *Points,* average of >10 independent experiments. Significance level was $P < 0.001$ at all points. **B,** the level of MPG expression in cells as measured by increasing MOI results in increased cell killing.

cells were treated with several alkylating DNA-damaging agents, and cell survival after treatment was monitored. The monofunctional alkylating agents MNNG, MNU, and DMS were used to treat MPG-overexpressing cells. These laboratory drugs, which are carcinogenic agents, primarily create different proportions of the MPG substrates N^7-methylguanine (82%, 70%, and 80%, respectively) and N^3-methyladenine (9%, 4%, and 16%, respectively), along with low levels of a host of other lesions (40), including highly toxic O^6-methylguanine lesions (9%, 11%, and 0.4%, respectively) not recognized by MPG. Multiple repeat MTS survival experiments show that cells expressing high levels of HA-MPG were significantly more sensitive to the cytotoxic effects of MNNG (Fig. 3A), MNU (Fig. 3B), and DMS (data not shown), with changes in the IC_{50} of 3.23 and 1.90, respectively. Sensitivity was verified by SRB and [^{3}H]thymidine assays (data not shown), which produced nearly identical results.

The clinical alkylating agent temozolomide is a recently developed chemotherapeutic that has been very effective in the treatment of astrocytoma, glioblastoma, brain

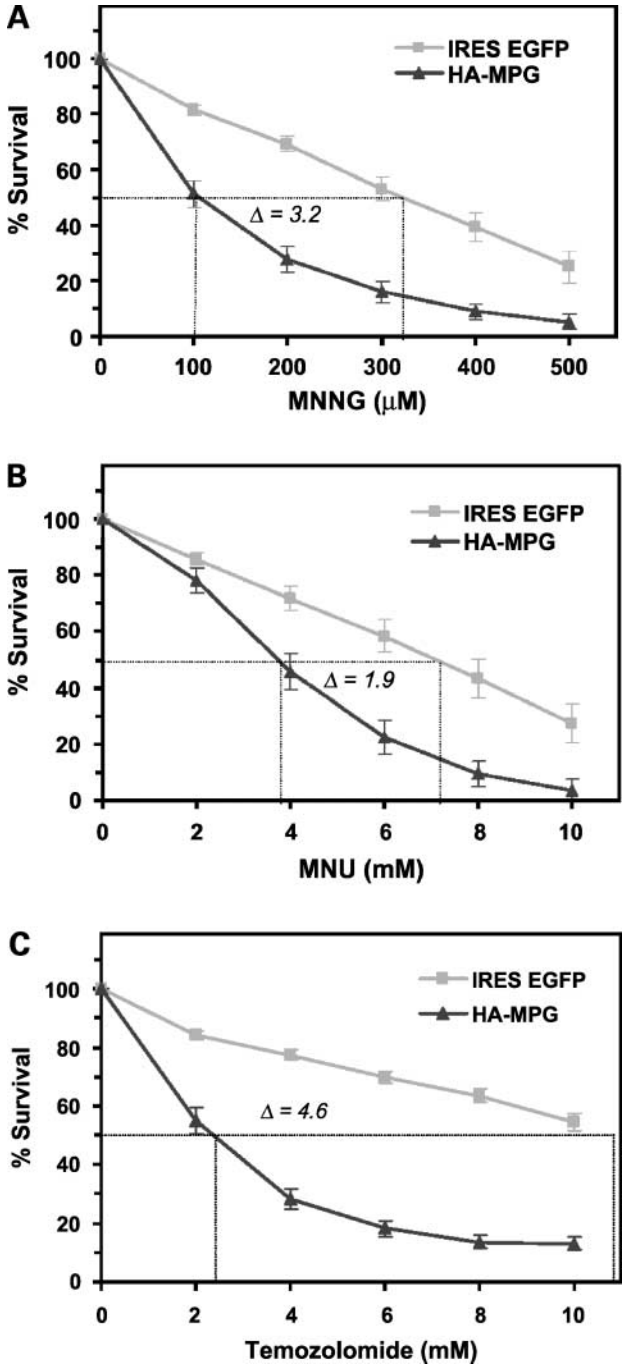

Figure 3. MTS survival assay results of adenovirally infected MDA-MB-231 breast cancer cells treated with MNNG, MNU, and temozolomide. **A,** HA-MPG-overexpressing cells are significantly more sensitive than vector-infected controls (decreased IC_{50} of 3.23-fold) to alkylation by MNNG. *Points,* average of >10 independent experiments. Differences were significant at $P < 0.001$ at all points. **B,** HA-MPG-overexpressing cells are significantly more sensitive than vector-infected controls (decreased IC_{50} of 2.0-fold) to alkylation by MNU. *Points,* average of 10 independent experiments. Differences were significant at $P < 0.001$ at all points. **C,** HA-MPG-overexpressing cells are significantly more sensitive than vector-infected controls (decreased IC_{50} of 4.5-fold) to alkylation by temozolomide. *Points,* average of >10 independent experiments. Significance level was $P < 0.001$ at all points.

metastases from other solid tumors, and malignant melanoma (41). Temozolomide is nonenzymatically hydrolyzed in solution to the active compound 3-methyl-(triazen-1-yl)imidazole-4-carboxamide. This same active compound is also produced by cytochrome activation of the clinical prodrug dacarbazine and is in the same class of compounds as MNNG and MNU. Temozolomide, unlike dacarbazine, does not require enzymatic activation by liver enzymes for activity. This not only simplifies drug metabolism and interactions in patients but also allows for the use of temozolomide in laboratory cell lines without requiring concomitant expression of cytochrome *P*450 enzymes. Like the laboratory alkylating agents tested for MPG-induced sensitivity, activated 3-methyl-(triazen-1-yl)imidazole-4-carboxamide from temozolomide or dacarbazine methylates DNA primarily at the N^7 and O^6 positions of guanine and N^3 position of adenine (70%, 5%, and 9%, respectively; ref. 42). This profile of damage makes temozolomide an excellent candidate for extending MPG overexpression-based sensitivity in cell lines to clinical chemotherapeutic agents. Results would have translational significance for both temozolomide and dacarbazine treatment.

Temozolomide was used to treat adenovirus-infected MDA-MB-231 cells, and it was determined that HA-MPG-overexpressing cells were extremely sensitive to temozolomide (4.58-fold decreased IC_{50} in >10 independent experiments) when compared with their vector control counterparts (Fig. 2C). This is the first time that MPG overexpression has been shown to influence the response of tumor cells to a clinical chemotherapeutic agent.

Measurement of Apoptosis in MPG-Overexpressing Cells Treated with Alkylators

To characterize and confirm the increased cell death occurring in MPG-overexpressing cells treated with alkylating agents, cells were assayed for apoptosis using Annexin V staining. Annexin V staining marks an early step in apoptosis and precedes the later loss of membrane integrity that occurs in the final stages of both apoptotic and necrotic cell death. Annexin V has been conjugated to several fluorochromes that allow flow cytometric detection of apoptotic cells. Because HA-MPG-overexpressing cells also co-overexpress the green fluorescent marker EGFP (emission wavelength 507 nm, detected in fluorescence channel FL-1), a PE-conjugated Annexin V that fluoresces in the red spectrum (emission wavelength 570 nm, analyzed with FL-2) was chosen to measure apoptosis. The vital dye 7-AAD, a standard flow cytometric viability probe with fluorescence detected in the far red (emission wavelength 660 nm, analyzed with FL-3), was chosen to distinguish viable from nonviable cells. These fluorescent markers allowed living cells undergoing apoptosis and dead MPG-overexpressing cells to be distinguished by flow cytometry.

Adenovirus-infected cells that had been treated with MMS were collected 6, 12, 18, and 25 hours after drug treatment and evaluated for apoptosis by Annexin V-PE/7-AAD staining. Flow cytometric results showed extreme sensitivity of HA-MPG-overexpressing cells to cytotoxicity from MMS (Fig. 4A; data shown are at 25 hours). Similar increases in cell death were seen in MPG-overexpressing cells treated with temozolomide (Fig. 4B). The number of dead HA-MPG cells progressively accrued significantly beyond vector control levels (Fig. 4A; upper right quadrants).

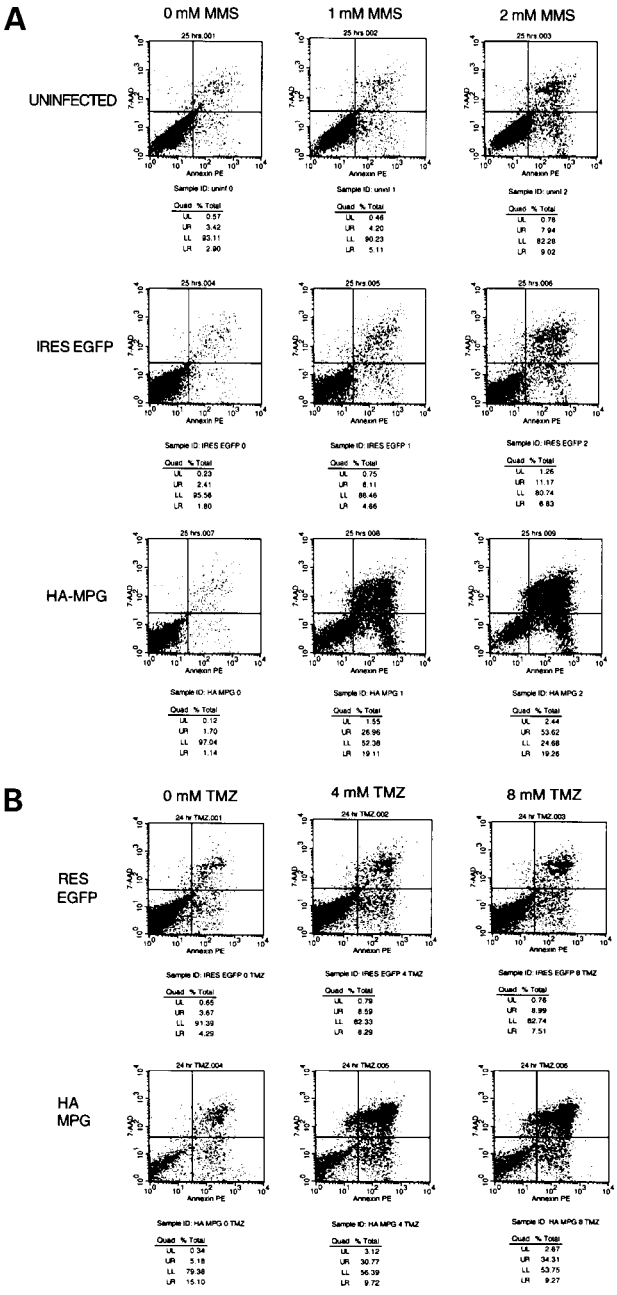

Figure 4. Assessment of apoptosis in adenovirally infected MDA-MB-231 breast cancer cells 25 hours after treatment with MMS (**A**) or temozolomide (*TMZ*; **B**) using Annexin V-PE/7-AAD staining followed by fluorescence-activated cell sorting analysis. HA-MPG-overexpressing cells (*HA-MPG*) are dramatically more sensitive than uninfected or vector-infected (*IRES2-EGFP*) control cells to apoptosis and total cytotoxicity induced by MMS or temozolomide.

This movement of cells from the lower left Annexin V-PE negative, 7-AAD negative quadrant through the lower right Annexin V-PE positive, 7-AAD negative quadrant (cells undergoing apoptosis) into the upper right Annexin V-PE positive, 7-AAD positive quadrant (cells undergoing apoptosis and dead cells), although not simultaneous, strongly suggests a killing pathway through apoptosis. A similar pattern was observed for temozolomide (Fig. 4B).

Because Annexin V staining can recognize cells that are undergoing programmed cell death, this assay was able to identify an even greater difference in cell survival between HA-MPG and vector-infected groups than was seen with previous viability assays. These experiments definitively show that HA-MPG-overexpressing cells are significantly more sensitive to the effects of alkylating agent chemotherapy than their vector control counterparts. Overexpressing cells also seem to more readily undergo apoptotic death in response to alkylating agent chemotherapy.

Measurement of BER Intermediates

The increased sensitivity of MPG-overexpressing cells to alkylating agents is believed to be the result of an imbalance in the BER pathway that leads to an amplification of toxic repair intermediates. To confirm the presence of increased levels of DNA repair intermediates in HA-MPG-overexpressing cells treated with MMS, the single-cell gel electrophoresis or comet assay was done (37). Cells were assayed 6 hours after treatment with MMS. The timing of the assay was done to precede any significant cell death as confirmed by Annexin V-PE/7-AAD staining (Fig. 4). Both neutral and alkaline comet assays were used to assess DNA damage in HA-MPG-overexpressing cells 6 hours after treatment with MMS. Results showed that HA-MPG-overexpressing cells had much higher levels of DNA damage in response to the alkylating agent MMS (Fig. 5A and B). Both alkaline and neutral comet assay results showed significantly increased comet tails in the HA-MPG group treated with MMS compared with vector control. The alkaline comet results indicate an increase in alkali labile sites (AP sites or single-strand breaks), whereas the neutral analysis measures double-strand breaks. Significant increases in the comet tails in the HA-MPG group treated with MMS showed that HA-MPG-infected cells produce AP sites, single-strand breaks, and/or double-strand breaks in response to MMS treatment (Fig. 5B). We have observed similar results with temozolomide (data not shown).

Sensitivity of HA-MPG-Overexpressing Cells to Alkylators in the Presence of the BER Inhibitor Methoxyamine

In imbalanced BER, increased MPG activity converts alkylated bases into toxic BER intermediates more rapidly than downstream enzymes can handle them. To show heightened production of AP sites and see whether HA-MPG-induced sensitivity could be potentiated by trapping AP sites, methoxyamine was used to treat cells exposed to the alkylating agents MMS and temozolomide. Methoxyamine is a BER inhibitor that specifically reacts with the C1 aldehyde of AP sites (43). Resulting methoxyamine-adducted AP sites are refractory to both APE1 cleavage activity and β-polymerase deoxyribophosphodiesterase/lyase activity (44, 45). This interruption to BER sensitizes cells to methylation with MMS or temozolomide by stabilizing the cytotoxic AP site intermediate (46). Incompletely repaired methoxyamine-blocked AP sites lead to increases in MMS or temozolomide-induced single-strand and double-strand breaks as well as cell death (47).

An AP site-blocking concentration of methoxyamine (10 mmol/L; ref. 48) was included in the growth medium of cells during and after treatment with alkylating agents. As had been observed previously, methoxyamine sensitized cells to alkylation. This effect was dramatically increased in the cells overexpressing HA-MPG, as methoxyamine greatly enhanced the alkylator sensitivity observed with imbalanced BER (Fig. 6) for both MMS (decreased IC_{50} of 5-fold) and temozolomide (decreased IC_{50} of >6-fold). This potentiation of cytotoxic sensitivity to alkylation by both MMS and temozolomide is consistent with a significantly increased production of AP sites in treated HA-MPG-overexpressing cells. It additionally suggests that stabilizing baseless sites with methoxyamine increases the cytotoxicity of imbalanced BER (49). Clearly, preventing the repair of AP sites through methoxyamine binding makes cells more sensitive to alkylation, and this effect is substantially greater in cells that are BER imbalanced. It is the conversion of toxic AP sites into irreparable toxic lesions that we believe is responsible for the sensitizing effect of methoxyamine.

Discussion

A large portion of cancer chemotherapy works by modifying the DNA of treated cells profoundly enough that responding cellular mechanisms disallow continued heredity and lead instead to cell death. Unfortunately, chemotherapy is unable to exclusively target tumor cells, and the additional death of normal cells limits the amount of therapy that can be tolerated (50). Many of the chemotherapeutic alterations made to DNA structure are handled by the DNA BER pathway (51). This pathway removes specific subtly damaged bases by processing them through baseless and strand-broken intermediates before restoring the DNA sequence. Enhanced MPG removal of damaged bases is thought to overwhelm the later rate-limiting stages of the pathway so that repair cannot be effectively completed and high levels of toxic intermediates signal cell death (3, 23, 24, 49, 52, 53).

We set out to explore this phenomenon of imbalanced repair as a possible approach for making tumor cells more sensitive to the effects of alkylating agent chemotherapy. To study the ability of MPG overexpression to sensitize tumor cells to the toxic effects of alkylation chemotherapy, a system for enhanced gene delivery and expression had to be established. Previous stable overexpression experiments showed that cells with high levels of MPG in the nucleus or mitochondria had moderate sensitivity to alkylation along with decreased cell survival in the absence of alkylating agent (24). MPG has been shown to excise

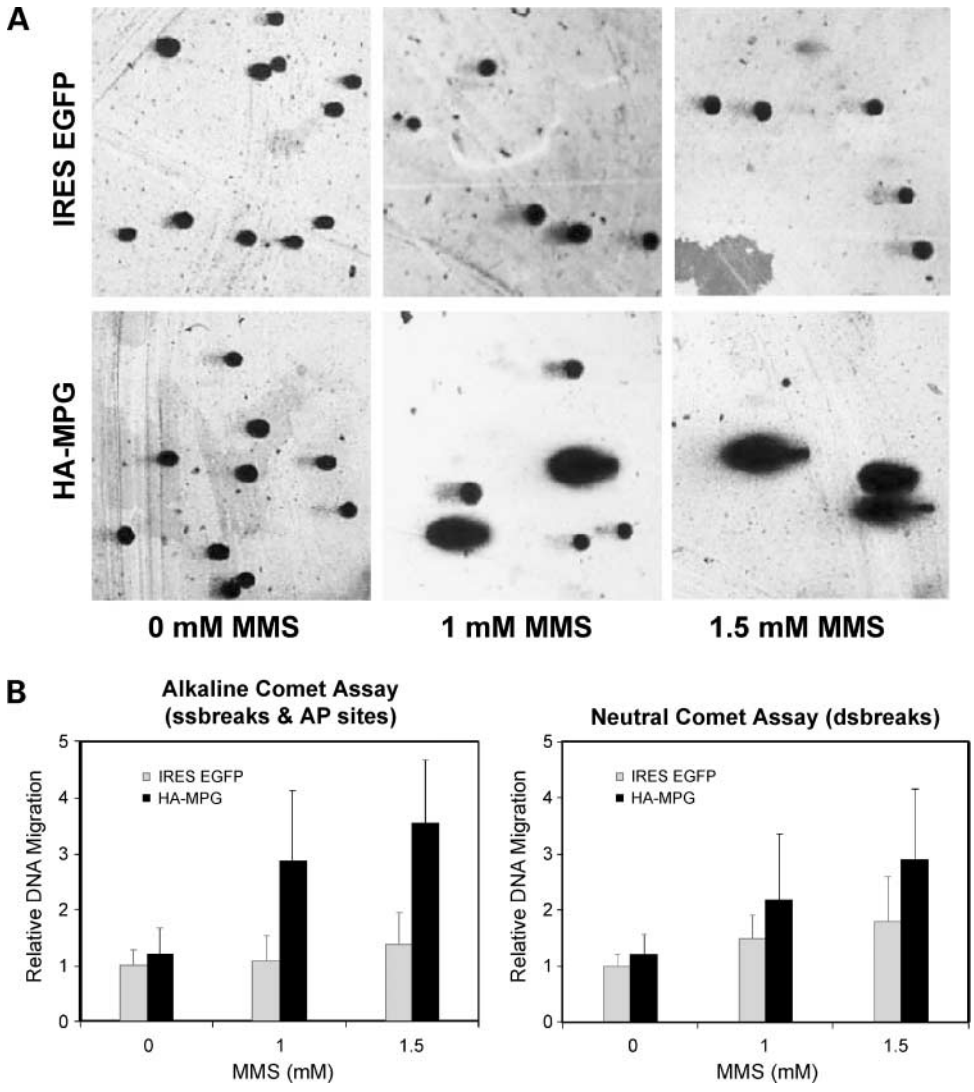

Figure 5. Alkaline and neutral single-cell comet assay. **A,** results demonstrating strong increases in DNA strand breaks and alkali labile sites in HA-MPG-overexpressing cells treated with MMS. Representative alkaline comet analysis in **A** and quantified data in **B** (*left panel*). Additionally, the comet assay was done under neutral conditions that result in only double-strand breaks being assessed (**B**, *right panel*). *Columns,* average comet tail length of 50 individual cells relative to untreated control cells; *bars,* SD. Single-strand breaks and labile sites (*left panel*) as well as the double strand breaks (*right panel*) are significantly greater in HA-MPG-overexpressing MDA-MB-231 cells confirmed by two-way ANOVA ($P < 0.001$).

undamaged guanine residues at a low but significant rate (26), and this promiscuous activity of the glycosylase during clonal selection may contribute to decreased cell survival of stable clones.

Adenoviral infection of MDA-MB-231 breast cancer cells resulted in high efficiency transduction and extremely high HA-MPG expression levels within 24 hours of exposure to modest concentrations (15 MOI) of adenovirus. Overexpressed HA-MPG protein correctly concentrated in the nucleus of infected cells and imparted dramatically increased MPG glycosylase activity. Significantly, adenovirus-infected HA-MPG-overexpressing cells had no decreased cell survival when compared with vector-infected or uninfected controls over the course of the experiments.

These results establish the utility of adenoviral-mediated overexpression for transiently delivering high levels of MPG into a human breast cancer cell line. This approach to MPG overexpression represents an improvement for establishing a BER imbalance not only because it affords higher levels of MPG but also because those levels can be achieved without the negative survival effects of long-term stable overexpression (i.e., mutation induction). These advantages are important both for studying MPG-induced sensitivity and for translating it into more clinically relevant tumor models.

Breast cancer cells overexpressing HA-MPG were found to be significantly more sensitive to cytotoxicity resulting from the alkylating agents MMS, MNNG, MNU, DMS, and the clinical antitumor agent temozolomide. These results verify the ability of increased MPG to sensitize cells to alkylation and provide further evidence for this overexpression-based imbalance of BER. The survival sensitivity achieved by these experiments was much greater than had been shown previously with MPG overexpression. This may be a result of the higher levels of MPG imparted by adenoviral infection or the fact that transient overexpression precludes the toxicity of extended high-level MPG activity during clonal selection, allowing a truer assessment of MPG-induced sensitivity. Whatever the case,

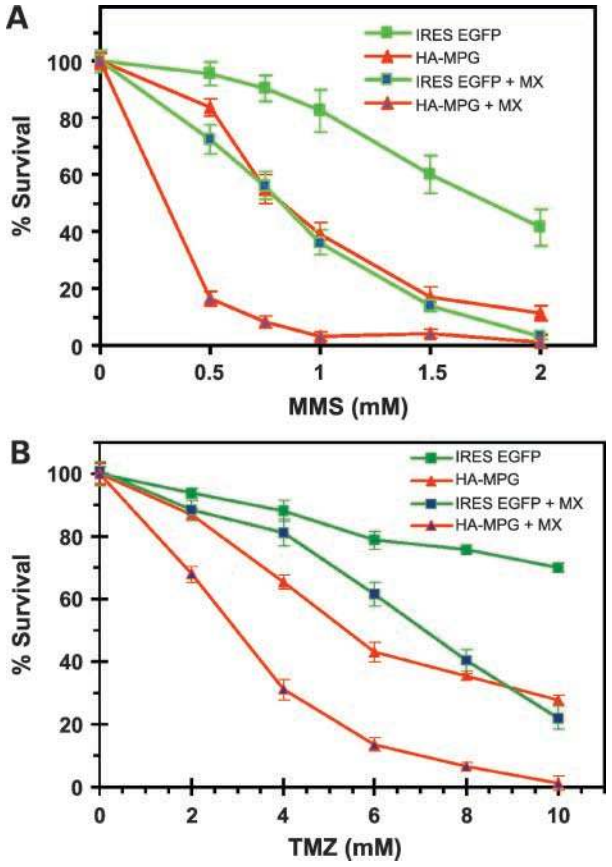

Figure 6. MTS survival assay results of adenovirally infected MDA-MB-231 breast cancer cells treated with MMS (**A**) or temozolomide (*TMZ*; **B**) and methoxyamine (*MX*; 10 mmol/L). Methoxyamine sensitizes cells to the effects of alkylation by MMS, but this effect is dramatically increased for HA-MPG-overexpressing cells for both MMS and temozolomide. Differences were significant at $P < 0.001$ between methoxyamine and/or HA-MPG versus HA-MPG + methoxyamine for both MMS and temozolomide. Methoxyamine and/or HA-MPG were also significantly different ($P < 0.001$) versus vector alone again for both MMS and temozolomide.

the adenoviral-mediated MPG overexpression employed in these studies reveals a more profound ability of increased MPG to sensitize cells to alkylation. This establishes MPG gene delivery as a feasible approach to be investigated for increasing tumor cell sensitivity toward alkylating agents.

The clinical alkylating agent temozolomide is a recently developed chemotherapeutic that has been very effective in the treatment of astrocytoma, glioblastoma, brain metastases from other solid tumors, and malignant melanoma (41). It has the distinction of being the first water-soluble and hence p.o. available chemotherapeutic capable of crossing the blood-brain barrier. Once administered, temozolomide is nonenzymatically hydrolyzed in solution to the active compound 3-methyl-(triazen-1-yl)imidazole-4-carboxamide. This same active compound is also produced by cytochrome activation of the clinical prodrug dacarbazine. Because they share a common active form, the clinical use of temozolomide has been guided by established uses for dacarbazine. By extension, temozolomide will likely be effective in the treatment of lymphomas and all soft tissue sarcomas such as fibrosarcoma, rhabdomyosarcoma, leiomyosarcoma, and neuroblastoma; recognized uses of dacarbazine have not yet expanded to temozolomide (54).

Temozolomide, unlike dacarbazine, does not require enzymatic activation by liver enzymes for activity. This not only simplifies drug metabolism and interactions in patients but also allows for the use of temozolomide in laboratory cell lines without requiring concomitant expression of cytochrome *P*450 enzymes. Like the laboratory alkylating agents tested for MPG-induced sensitivity, activated 3-methyl-(triazen-1-yl)imidazole-4-carboxamide from temozolomide or dacarbazine methylates DNA primarily at the N^7 and O^6 positions of guanine and N^3 position of adenine (70%, 5%, and 9%, respectively; ref. 42). This profile of damage makes temozolomide an excellent candidate for extending MPG overexpression-based sensitivity in cell lines to clinical chemotherapeutic agents. Results presented in this report are the first to show that MPG overexpression can increase tumor cell sensitivity to a clinical chemotherapeutic temozolomide. The successful application of a BER imbalance toward increasing the efficacy of clinical chemotherapeutics begins to extend MPG gene delivery into this clinical realm.

To appropriately apply imbalanced repair in sensitizing cancer to chemotherapy, it is important to understand how MPG-mediated sensitivity occurs. To begin to establish the mechanisms by which heightened MPG activity leads to increased cell death, apoptosis was measured following alkylating agent exposure. Cells infected with HA-MPG-containing adenovirus underwent apoptosis at a higher frequency, and the percentage that eventually died from alkylating agent exposure was significantly greater than vector-infected controls. In fact, because an assessment of apoptosis is able to recognize cells that have entered—in addition to those that have completed—programmed cell death, these results showed even higher survival differentials than earlier sensitivity experiments. These higher estimates of sensitivity imply that the ultimate effects of MPG overexpression may be even greater than earlier cell survival estimates had shown.

Increased induction of apoptosis in those cells with enhanced handling of alkylated DNA damage implies that amplified MPG action in response to alkylation increasingly initiates the program for cell suicide. It seems probable that increased baseless and strand-broken BER intermediates blamed for the toxicity of an imbalance are potent triggers that activate the apoptotic machinery. This would mean that such structural disruptions to the genetic material are established signals representing intolerable change that must be avoided even at the cost of self-sacrifice. Although these intermediates and the disruptions that they incite also push cells down alternative pathways leading to necrotic cell death, it is apparent that the apoptotic pathway plays a role in MPG-induced cell death.

The sensitivity to alkylation caused by MPG overexpression is believed to result from imbalanced repair,

wherein high levels of glycosylase activity excise damaged bases and create repair intermediates that cannot be adequately handled by downstream enzymes. A role for toxic AP sites and single-strand breaks in this process has long been postulated but never directly shown in mammalian or tumor cells. The alkaline comet assay established that these BER intermediates were significantly increased in MPG-overexpressing cells treated with drug. Although unable to distinguish between them, the results show that AP sites and/or single-strand breaks are elevated in overexpressing cells prior to evidence of apoptosis or necrosis by Annexin V/7-AAD staining, indicating that these increases are not secondary to cell death. As a direct measurement of the presence of increased BER intermediates in cells with higher expression of MPG, these data give compelling support to the model of imbalanced repair. It additionally endorses a potential role for AP sites and/or single-strand breaks in contributing to the alkylation sensitivity caused by MPG overexpression. Additionally, an increase in double-strand breaks was observed. Whether this is the actual killing event is unknown at this time. Additionally, how these double-strand breaks are created is unknown, but they could be due either to the juxtaposition of AP sites, the result of AP sites positioned in topoisomerase II cleavage sites effectively poisoning the relegation of topoisomerase II–cleaved DNA, or another mechanism that has yet to be determined. This is an area undergoing investigation by our group and others (55–58).

To further explore the cellular consequences of AP sites, MPG-sensitized cells were exposed to the BER inhibitor methoxyamine, a compound that covalently reacts with AP sites (43) and makes them refractory to repair by APE1 and β-polymerase (44, 45). Cells were exposed to methoxyamine during and after treatment with alkylating agents to turn AP sites into persistent irreparable lesions. This action of methoxyamine is known to make cells more sensitive to the effects of alkylation (47, 59–61), but its effects on MPG-overexpressing cells were even more dramatic. The significantly increased sensitivity created by methoxyamine in MPG-transduced cells for both MMS and temozolomide is evidence for increased production of AP sites and suggests that making those AP sites permanent increases their toxicity. This underscores the role of baseless residues in MPG-induced sensitivity and indicates that they may lead directly to toxicity even without being processed to single-strand breaks (49).

The concept of AP sites as an effective ''cellular poison'' has been strengthened by work in *Saccharomyces cerevisiae* that showed delivery of high levels of human MPG or the yeast homologue (Mag1) into a strain deficient for Mag1, resulting in an increased sensitization of yeast to killing by MMS (62). The sensitivity caused by Mag1 overexpression was suppressed by yeast AP endonuclease (Apn1) co-overexpression, lending strength to the notion of an imbalanced repair and its reliance on increased AP sites. Mag1 overexpression also significantly increased spontaneous mutation frequency, but this effect was dramatically lower with MPG overexpression. Similarly, overexpression of the rat MPG homologue rendered Chinese hamster ovary cells more sensitive to cytotoxicity of MMS. The involvement of AP sites in this sensitivity was inferred from the observation that overexpressing cells showed a 2-fold increase in AT base pair mutational changes, potentially resulting from increased alkylated base removal and AP site replication-induced mutagenesis (63). All of this experimental evidence suggests that the toxic nature of BER intermediates, particularly AP sites, can be exploited to sensitize cells to DNA change by increasing the levels of MPG.

Conclusions

The use of adenovirus for the delivery of MPG into human breast cancer cells not only employs a clinically relevant gene therapy vector (64) but also affords high-level gene expression—a necessary feature, as BER imbalance relies on MPG expression levels. Additionally, adenoviral transgenes are not integrated into the host cell genome but remain as epigenetic elements that are diluted with cell division and eventually lost (65). Because of the potentially mutagenic and cytotoxic effects of long-term MPG overexpression stemming from cumulative although low-level normal base removal, such transient overexpression is preferred.

Our results show that MPG-overexpressing cells are significantly more sensitive to the alkylating agents MMS, MNNG, MNU, DMS, and the clinical chemotherapeutic agent temozolomide. Sensitivity is further increased through coadministration of the BER inhibitor methoxyamine. The methoxyamine reduction of cell survival leads us to believe that stabilizing AP sites is an effective means of cellular toxicity. Cells were determined to be dying through an apoptotic pathway. It also seems that there is a significant increase in the number of AP sites and/or single-strand and double-strand breaks in overexpressing cells as determined, albeit indirectly, using the comet assay, confirming a MPG-driven accumulation of toxic BER intermediates. These data establish transient MPG overexpression as a potential therapeutic approach for increasing cellular sensitivity to alkylating agent chemotherapy.

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